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בקשה לפטנט
Application for Patent

אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
I (Name and address of applicant, and in case of body corporate-place of incorporation)

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בעל אמצאה מכח Assignment שמה הוא
Owner, by virtue of an invention the title of which is

(בעברית) (Hebrew)
מודולטורים של פקטור הקשור לקולטן TNF (TRAF), הכנתם והשימוש בהם

(באנגלית) (English)
MODULATORS OF TNF RECEPTOR ASSOCIATED FACTOR (TRAF), THEIR PREPARATION AND USE

מבקש בזאת כי ינתן לי עליה פטנט
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• בקשת חלוקה — Application of Division	• בקשת פטנט מוסף — Application for Patent Addition	• דרישה דין קדימה Priority Claim		
מבקשת פטנט from Application	• לבקשה/לפטנט to Patent/Appl.	מספר/סימן Number/Mark	תאריך Date	מדינת האגוד Convention Country
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dated מיום	dated מיום			

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MODULATORS OF TNF RECEPTOR ASSOCIATED FACTOR (TRAF), THEIR PREPARATION AND USE

מודולטורים של פקטור הקשור לקולטן TNF (TRAF), הכנתם והשימוש בהם

Yeda Research and Development Co. Ltd.

ידע חברה למחקר ופתוח בע"מ

Field of the Invention

The present invention concerns DNA sequences encoding proteins capable of binding to TRAF2, and the proteins encoded thereby, and the use of said proteins and DNA sequences in the treatment or prevention of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which said proteins bind.

Background of the Invention

The Tumor Necrosis Factor/ Nerve Growth Factor (TNF/NGF) receptor superfamily is defined by structural homology between the extracellular domains of its members (Bazan, 1993; Beutler and van Huffel, 1994; Smith et al., 1994). Except for two receptors, the p55 TNF receptor and Fas/APO1, the various members of this receptor family do not exhibit clear similarity of structure in their intracellular domains. Nevertheless, there is much similarity of function between the receptors, indicating that they share common signaling pathways. One example for this similarity is the ability of several receptors of the TNF/NGF family to activate the transcription factor NF-kB. This common ability was ascribed to a capability of a cytoplasmic protein that activates NF-kB, TNF Receptor Associated Factor 2 (TRAF2) to bind to the structurally-dissimilar intracellular domains of several of the receptors of the TNF/NGF family. By what mechanisms does TRAF2 act and how is its responsiveness to the different receptors to which it binds coordinated is not known.

TRAF2 is a member of a recently described family of proteins called TRAF that includes so far 3 proteins identified as TRAF1, TRAF2 (Rothe, M., Wong, s.c., Henzel, W.J. and Goeddel, D (1994) Cell 78:681-692; PCT published application WO 95/33051) and TRAF3 (Cheng, G. et al. (1995)).

All proteins belonging to the TRAF family share high degree of amino acid identity in their C-terminal domains, while their N-terminal domains may be unrelated. As shown in a schematic illustration of TRAF2 (Fig. 1), the molecule contains a ring finger motif and two TFIIIA-like zinc finger motifs at its N-terminal area. The C-terminal half of the molecule includes a region known as the "TRAF domain" containing a potential leucine zipper region extending between amino acids 264 - 358 (called N-TRAF), and another part towards the carboxy end of the domain between amino acids 359 - 501 (called C-TRAF) which is responsible for TRAF binding to the receptors and to other TRAF molecules to form homo- or hetrodimers.

Activation of the transcription factor NF-kB is one manifestation of the signaling cascade initiated by some of the TNF/NGF receptors and mediated by TRAF2. NF-kB comprises members of a family of dimer-forming proteins with homology to the Rel oncogene which, in their dimeric form, act as transcription factors. These factors are ubiquitous and participate in regulation of the expression of multiple genes. Although initially identified as a factor that is constitutively present in B cells at the stage of Igk light chain expression, NF-kB is known primarily for its action as an inducible transcriptional activator. In most known cases NF-kB behaves as a primary factor, namely the induction of its activity is by activation of pre-existing molecules present in the cell in their inactive form, rather than its de-novo synthesis which in turn relies on inducible transcription factors that turn-on the NF-kB gene. The effects of NF-kB are highly pleiotropic. Most of these numerous effects share the common features of being quickly induced in response to an extracellular stimulus. The majority of the NF-kB-activating agents are inducers of immune defense, including components of viruses and bacteria, cytokines that regulate immune response, UV light and others. Accordingly, many of the genes regulated by NF-kB contribute to immune defense (see Blank et al., 1992; Grilli et al., 1993; Baeuerle and Henkel, 1994, for reviews).

One major feature of NF-kB-regulation is that this factor can exist in a cytoplasmic non-DNA binding form which can be induced to translocate to the nucleus, bind DNA and activate transcription. This dual form of the NF-kB proteins is regulated by I-kB - a family of proteins that contain repeats of a domain that has initially been discerned in the erythrocyte protein ankyrin (Gilmore and Morin, 1993). In the unstimulated form, the NF-kB dimer occurs in association with an I-kB molecule which imposes on it cytoplasmic location and prevents its interaction with the NF-kB-binding DNA sequence and activation of

transcription. The dissociation of I-kB from the NF-kB dimer constitutes the critical step of its activation by many of its inducing agents (DiDonato et al., 1995). Knowledge of the mechanisms that are involved in this regulation is still limited. There is also just little understanding of the way in which cell specificity in terms of responsiveness to the various NF-kB-inducing agents is determined.

One of the most potent inducing agents of NF-kB is the cytokine tumor necrosis factor (TNF). There are two different TNF receptors, the p55 and p75 receptors. Their expression levels vary independently among different cells (Vandenabeele et al., 1995). The p75 receptor responds preferentially to the cell-bound form of TNF (TNF is expressed both as a beta-transmembrane protein and as a soluble protein) while the p55 receptor responds just as effectively to soluble TNF molecules (Grell et al., 1995). The intracellular domains of the two receptors are structurally unrelated and bind different cytoplasmic proteins. Nevertheless, at least part of the effects of TNF, including the cytocidal effect of TNF and the induction of NF-kB, can be induced by both receptors. This feature is cell specific. The p55 receptor is capable of inducing a cytocidal effect or activation of NF-kB in all cells that exhibit such effects in response to TNF. The p75-R can have such effects only in some cells. Others, although expressing the p75-R at high levels, show induction of the effects only in response to stimulation of the p55-R (Vandenabeele et al., 1995). Apart from the TNF receptors, various other receptors of the TNF/NGF receptor family: CD30 (McDonald et al., 1995), CD40 (Berberich et al., 1994; Lalmanach-Girard et al., 1993), the lymphotoxin beta receptor and, in a few types of cells, Fas/APO1 (Rensing-Ehl et al., 1995), are also capable of inducing activation of NF-kB. The IL-1 type I receptor, also effectively triggering NF-kB activation, shares most of the effects of the TNF receptors despit the fact that it has no structural similarity to them.

The activation of NF-kB upon triggering of these various receptors results from induced phosphorylation of its associated I-kB molecules. This phosphorylation tagges I-kB to degradation, which most likely occurs in the proteasome. The nature of the kinase that phosphorylates I-kB, and its mechanism of activation upon receptor triggering is still unknown. However, in the recent two years some knowledge has been gained as to the identity of three receptor-associated proteins that appear to take part in initiation of the phosphorylation (See diagrammatic illustration in Figure 2). A protein called TRAF2, initially cloned by D. Goeddel and his colleagues (Rothe et al., 1994), seems to play a central

role in NF-kB-activation by the various receptors of the TNF/NGF family. The protein, which when expressed at high levels can by itself trigger NF-kB activation, binds to activated p75 TNF-R (Rothe et al., 1994), lymphotoxin beta receptor (Mosialos et al., 1995), CD40 (Rothe et al., 1995a) and CD-30 (unpublished data) and mediates the induction of NF-kB by them. TRAF2 does not bind to the p55 TNF receptor nor to Fas/APO1, however, it can bind to a p55 receptor-associated protein called TRADD and TRADD has the ability to bind to a Fas/APO1-associated protein called MORT1 (or FADD). These associations apparently allow the p55 TNF receptor and Fas/APO1 to trigger NF-kB activation (Hsu et al., 1995; Boldin et al., 1995; Chinnalyan et al., 1995; Varfolomeev et al., 1996; Hsu et al., 1996). The triggering of NF-kB activation by the IL-1 receptor occurs independently of TRAF2 and may involve a recently-cloned IL-1 receptor-associated protein-kinase called IRAK (Croston et al., 1995).

By what mechanism does TRAF2 act is not clear. Several cytoplasmic molecules that bind to TRAF2 have been identified (Rothe et al., 1994; Rothe et al., 1995b). However, the information of these molecules does not provide any clue as to the way by which TRAF2, which by itself does not posses any enzymatic activity, triggers the phosphorylation of I-kB. There is also no information yet of mechanisms that dictate cell-specific pattern of activation of TRAF2 by different receptors, such as observed for the induction of NF-kB by the two TNF receptors.

Summary of the Invention

The present invention provides DNA sequences encoding for proteins that are capable of binding to a tumor necrosis factor receptor-associated factor (TRAF) molecule.

By one aspect, the present invention provides DNA encoding a protein capable of binding to TRAF2 selected from the group consisting of:

(a) a cDNA sequence of the herein designated clone 9 comprising the nucleotide sequence depicted in Fig 3a;

(b) a cDNA sequence of the herein designated clone 10 comprising the nucleotide sequence depicted in Fig 4a;

(c) a cDNA sequence of the herein designated clone 15 comprising the nucleotide sequence depicted in Fig 5a;

(d) a fragment of a sequence (a)-(c) which encodes a biologically active protein capable of binding to least the 222-501 amino acid sequence of TRAF2;

(e) a DNA sequence capable of hybridization to a sequence of (a)-(d) under moderately stringent conditions and which encodes a biologically active protein capable of binding to at least the 222-501 amino acid sequence of TRAF2; and

(f) a DNA sequence which is degenerate as a result of the genetic code to the DNA sequences defined in (a)-(e) and which encodes a biologically active protein capable of binding to at least the 222-501 amino acid sequence of TRAF2.

In prefered embodiments, the DNA sequence is selected from the sequences of the herein designated cDNA clones 9, 10 and 15, most preferable clone 10 that encodes a protein, herein designated NMP1 (NF-κB modulating protein 1), that also modulates NF-κB activity.

In another aspect, the invention provides proteins or polypeptides encoded by the DNA coding sequences of the invention, the analogs and derivatives of said proteins and polypeptides, provided that they are capable of binding to TRAF2, preferably to at least the 222-501 amino acid sequence of TRAF2, thereby mediating or modulating the signaling process in which TRAF2 is involved.

In yet another aspects, the invention provides a vector comprising a DNA sequence according to the invention which is capable of being expressed in host cells selected from prokaryotic and eukaryotic cells, and the transformed prokaryotic and eukaryotic cells containing said vector.

The invention also provides a method for producing a protein encoded by a DNA sequence according to the invention, and analogs and derivatives thereof, which comprises growing the above mentioned transformed host cells under conditions suitable for the expression of said protein, effecting post-translational modification, if necessary, for extraction of said protein, and extracting said protein from the culture medium or from cell extracts of said transformed host cells.

In a further aspect, the invention provides antibodies raised against a protein or polypeptide of the invention.

In a different aspect, the invention provides the following screening methods:

(i) A method for screening of a ligand capable of binding to a protein according to the invention, comprising contacting an affinity chromatography matrix to which said protein is attached with a cell extract whereby the ligand is bound to said matrix, and eluting, isolating and analyzing said ligand.

(ii) A method for screening of a DNA sequence coding for a ligand capable of binding to a protein according to the invention, comprising applying the yeast two-hybrid procedure in which a sequence encoding said protein is carried by one hybrid vector and sequences from a cDNA or genomic DNA library are carried by the second hybrid vector, transforming yeast host cells with said vectors, isolating the positively transformed cells, and extracting said second hybrid vector to obtain a sequence encoding said ligand.

In the embodiment of the invention is also a method for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a protein according to the invention binds, said method comprising administering to a patient in need an effective amount of a protein according to the invention or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein with TRAF2 or any other molecule to which said protein binds. In a prefered embodiment said protein of the invention administered to the patient in need is NMP1, or a DNA molecule coding therefor.

In yet another embodiment, the invention concerns a pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a protein according to the invention binds, said composition comprising an effective amount of a protein according to the invention or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein with TRAF2 or any other molecule to which said protein binds. In a prefered embodiment said pharmaceutical composition comprising an effective amount of NMP1 or a DNA molecule coding therefor.

Brief Description of the Drawings

Fig. 1 shows a diagramatic illustration of the structure of the TRAF2 molecule.

Fig. 2 shows a schematic diagram illustrating the known proteins involved in NF-kB activation.

Figs. 3a-b show partial and preliminary nucleotide sequence of the 5' end of clone 9 (a) and the deduced amino acid sequenced encoded thereby (b). Places of an uncertainty are marked by N for an undefined nucleotide, and by X for an undefined amino acid residue.

Figs. 4a-b show partial and preliminary nucleotide sequence of clone 10 (a) and the deduced amino acid sequence encoded thereby (b). Places of an uncertainty are marked by N for an undefined nucleotide, and by X for an undefined amino acid residue.

Figs. 5a-b show the preliminary nucleotide sequence of clone 15 (a) and the deduced amino acid sequence encoded thereby (b). Places of an uncertainty are marked by N for an undefined nucleotide, and by X for an undefined amino acid residue. A stop codon is marked by an asterisk.

Fig. 6 shows an alignment of the sequence of protein NMP1 with the sequence of the mouse protein kinase mMEKK (mouse MAPK or ERK Kinase Kinase). The regions corresponding to the conserved motifs I to XI in protein kinases are marked.

Fig. 7 shows the results of a gel retardation assay demonstrating that NMP1 blocks NFkB activation by p75-receptor.

Figs. 8a-b show the results of Luciferase (reporter gene) assays demonstrating that clone 10 has an inhibitory effect over basal NFkB activity (Fig. 8a), and that it also blocks NFkB activation by p75-receptor (Fig. 8b).

Detailed Description of the Invention

The present invention relates to DNA sequences encoding proteins capable of binding to a tumor necrosis factor receptor-associated factor (TRAF) molecule, and the proteins encoded thereby. In a prefered way, the present invention concerns cDNA sequences herein designated clone 9, clone 10 and clone 15 (depicited in Figs. 3a, 4a and 5a, respectively), which encode for proteins capable of binding to TRAF2, and the proteins encoded by those DNA sequences. The DNA and the deduced amino acid sequences of the three clones according to the invention represent new sequences; they do not appear in the 'GENEBANK' or 'PROTEIN BANK' data banks of DNA or amino acid sequences.

Whithin the scope of the present invention are also fragments of the above mentioned DNA sequences and DNA sequences capable of hybridization to those sequences or part of them, under moderately stringent conditions, provided they encode a biologically active protein or polypeptide capable of binding to at least the 222-501 amino acid sequence of TRAF2.

The present invention also concerns a DNA sequence which is degenerated as a result of the genetic code to the above mentioned DNA sequences and which encodes a biologically

active protein or polypeptide capable of binding to at least the 222-501 amino acid sequence of TRAF2.

Thus, the present invention concerns the DNA sequences encoding biologically active analogs, fragments and derivatives of thereof, and the analogs, fragments and derivatives of the proteins encoded thereby. The preparation of such analogs, fragments and derivatives is by standard procedures (see for example, Sambrook et al., 1989) in which in the DNA encoding sequences, one or more codons may be deleted, added or substituted by another, to yield encoded analogs having at least a one amino acid residue change with respect to the native protein. Acceptable analogs are those which retain at least the capability of binding to TRAF2 with or without mediating any other binding or enzymatic activity, e.g. analogs which bind TRAF2 but do not signal, i.e. do not bind to a further downstream protein or other factor, or do not catalyze a signal-dependent reaction. In such a way analogs can be produced which have a so-called dominant-negative effect, namely, an analog which is defective either in binding to TRAF2, or in subsequent signaling following such binding. Such analogs can be used, for example, to inhibit the CD40 or p75 TNF receptor effects by competing with the natural TRAF2-binding proteins. Likewise, so-called dominant-positive analogs may be produced which would serve to enhance the TRAF2 effect. These would have the same or better TRAF2-binding properties and the same or better signaling properties of the natural TRAF2-binding proteins. In an analogous fashion, biologically active fragments of the clones of the invention may be prepared as noted above with respect to the preparation of the analogs. Suitable fragments of the DNA sequences of the invention are those which encode a protein or polypeptide retaining the TRAF2 binding capability or which can mediate any other binding or enzymatic activity as noted above. Accordingly, fragments of the encoded proteins of the invention can be prepared which have a dominant-negative or a dominant-positive effect as noted above with respect to the analogs. Similarly, derivatives may be prepared by standard modifications of the side groups of one or more amino acid residues of the proteins, their analogs or fragments, or by conjugation of the proteins, their analogs or fragments, to another molecule e.g. an antibody, enzyme, receptor, etc., as are well known in the art.

All the above mentioned modifications are in the scope of the invention provided they preserved the ability of the encoded proteins or polypeptides or their analogs and derivatives thereof, to bind at least the 222-501 amino acid sequence of TRAF2.

All the proteins and polypeptides of the invention by virtue of their capability to bind to TRAF2, are considered as mediators or modulators of TRAF2 signaling. As such, said molecules of the invention have a role in, for example, the signaling process in which the binding of TRAF2 ligand to CD30, CD40, lymphotoxin beta (LT-β) receptor, p55 or p75 TNF receptors leads to activation of the transcription factor NF-κB. Particularly interesting is protein NMP1 encoded by clone 10 of the invention; a detailed sequence analysis of this clone disclosed encoded amino acid sequences corresponding to I - XI conserved motifs characteristic to Ser/Thr protein kinases, thus assigning a poteintial function to this protein.

The new clones proteins, their analogs, fragments and derivatives have a number of possible uses, for example:

(i) They may be used to mimic or enhance NFκB activity, the function of TRAF2 and the receptors to which they bind, in situations where an enhanced function is desired such as in anti-tumor or immuno-stimulatory applications where the TRAF2- induced effects are desired. In this case the proteins of the invention, their analogs, fragments or derivatives, which enhance the TRAF2 or receptors effects, may be introduced to the cells by standard procedures known per se. For example, as the proteins encoded by the DNA clones of the invention are intracellular and they should be introduced only into the cells where the TRAF2 effect is desired, a system for specific introduction of these proteins into the cells is necessary. One way of doing this is by creating a recombinant animal virus e.g. one derived from Vaccinia, to the DNA of which the following two genes will be introduced: the gene encoding a ligand that binds to cell surface proteins specifically expressed by the cells e.g. ones such as the AIDs (HIV) virus gp120 protein which binds specifically to some cells (CD4 lymphocytes and related leukemias) or any other ligand that binds specifically to cells carrying a receptor that binds TRAF2, such that the recombinant virus vector will be capable of binding such cells; and the gene encoding the proteins of the invention. Thus, expression of the cell-surface-binding protein on the surface of the virus will target the virus specifically to the tumor cell or other receptor- carrying cell, following which the proteins encoding sequences will be introduced into the cells via the virus, and once expressed in the cells will result in enhancement of the receptor or TRAF2 effect leading to a desired immuno-stimulatory effect in these cells. Construction of such recombinant animal virus is by standard procedures (see for example, Sambrook et al., 1989). Another possibility is to introduce the

sequences of the encoded proteins in the form of oligonucleotides which can be absorbed by the cells and expressed therein.

(ii) They may be used to inhibit the NFκB activity, the effects of TRAF2 or of the receptor that binds it, e.g. in cases such as tissue damage as in AIDS, septic shock or graft-vs.-host rejection, in which it is desired to block the induced intracellular signaling. In this situation it is possible, for example, to introduce into the cells, by standard procedures, oligonucleotides having the anti-sense coding sequence for the proteins of the invention, which would effectively block the translation of mRNAs encoding the proteins and thereby block their expression and lead to the inhibition of the undesired effect. Alternatively, other oligonucleotides may be used; oligonucleotides that preserved their ability to bind to TRAF2 in a way that interfers with the binding of other molecules to this protein, while at the same time do not mediate any activation or modulation of this molecule. Having these characteristics, said molecules can disrupt the interaction of TRAF2 with its natural ligand, therefor acting as inhibitors capable of abolishing effects mediated by TRAF2, such as NF-κB activation, for example. Such oligonucleotides may be introduced into the cells using the above recombinant virus approach, the second sequence carried by the virus being the oligonucleotide sequence.

Another possibility is to use antibodies specific for the proteins of the invention to inhibit their intracellular signaling activity.

Yet another way of inhibiting the undesired effect is by the recently developed ribozyme approach. Ribozymes are catalytic RNA molecules that specifically cleave RNAs. Ribozymes may be engineered to cleave target RNAs of choice, e.g. the mRNAs encoding the proteins of the invention. Such ribozymes would have a sequence specific for the mRNA of the proteins and would be capable of interacting therewith (complementary binding) followed by cleavage of the mRNA, resulting in a decrease (or complete loss) in the expression of the proteins, the level of decreased expression being dependent upon the level of ribozyme expression in the target cell. To introduce ribozymes into the cells of choice (e.g. those carrying the TRAF2 binding proteins) any suitable vector may be used, e.g. plasmid, animal virus (retrovirus) vectors, that are usually used for this purpose (see also (i) above, where the virus has, as second sequence, a cDNA encoding the ribozyme sequence of choice). (For reviews, methods etc. concerning ribozymes see Chen et al., 1992; Zhao and Pick, 1993).

(iii) They may be used to isolate, identify and clone other proteins which are capable of binding to them, e.g. other proteins involved in the intracellular signaling process that are downstream of TRAF2. For example, the DNA sequences encoding the proteins of the invention may be used in the yeast two-hybrid system in which the encoded proteins will be used as "bait" to isolate, clone and identify from cDNA or genomic DNA libraries other sequences ("preys") encoding proteins which can bind to the clones proteins. In the same way, it may also be determined whether the proteins of the present invention can bind to other cellular proteins, e.g. other receptors of the TNF/NGF superfamily of receptors.

(iv) The encoded proteins, their analogs, fragments or derivatives may also be used to isolate, identify and clone other proteins of the same class i.e. those binding to TRAF2 or to functionally related proteins, and involved in the intracellular signaling process. In this application the above noted yeast two-hybrid system may be used, or there may be used a recently developed system employing non-stringent Southern hybridization followed by PCR cloning (Wilks et al., 1989).

(v) Yet another approach to utilize the encoded proteins of the invention, their analogs, fragments or derivatives is to use them in methods of affinity chromatography to isolate and identify other proteins or factors to which they are capable of binding, e.g., proteins related to TRAF2 or other proteins or factors involved in the intracellular signaling process. In this application, the proteins, their analogs, fragments or derivatives of the present invention, may be individually attached to affinity chromatography matrices and then brought into contact with cell extracts or isolated proteins or factors suspected of being involved in the intracellular signaling process. Following the affinity chromatography procedure, the other proteins or factors which bind to the proteins, their analogs, fragments or derivatives of the invention, can be eluted, isolated and characterized.

(vi) As noted above, the proteins, their analogs, fragments or derivatives of the invention may also be used as immunogens (antigens) to produce specific antibodies thereto. These antibodies may also be used for the purposes of purification of the proteins of the invention either from cell extracts or from transformed cell lines producing them, their analogs or fragments. Further, these antibodies may be used for diagnostic purposes for identifying disorders related to abnormal functioning of the receptor system in which they function, e.g., overactive or underactive TRAF2- induced cellular effects. Thus, should such disorders be related to a malfunctioning intracellular signaling system involving the proteins

of the invention, such antibodies would serve as an important diagnostic tool. The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, anti-idiotypic (anti-Id) antibodies to antibodies that can be labeled in soluble or bound form, as well as fragments thereof, such as, for example, Fab and $F(ab')_2$ - fragments lacking the Fc fragment of intact antibody, which are capable of binding antigen.

(vii) The antibodies, including fragments of antibodies, useful in the present invention may be used to quantitatively or qualitatively detect the clones of the invention in a sample, or to detect presence of cells which express the clones of the present invention. This can be accomplished by immunofluorescence techniques employing a fluorescently labeled antibody coupled with light microscopic, flow cytometric, or fluorometric detection.

The antibodies (or fragments thereof) useful in the present invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of the clones of the present invention. *In situ* detection may be accomplished by removing a histological specimen from a patient, and providing the labeled antibody of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the clones, but also its distribution on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Such assays for the clones of the present invention typically comprises incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells such as lymphocytes or leukocytes, or cells which have been incubated in tissue culture, in the presence of a detectably labeled antibody capably of identifying the encoded proteins, and detecting the antibody by any of a number of techniques well known in the art.

(viii) The encoded proteins of the invention may also be used as indirect modulators of a number of other proteins by virtue of their capability of binding to other intracellular proteins, which other intracellular proteins directly bind yet other intracellular proteins or an intracellular domain of a transmembrane protein.

For the purposes of modulating these other intracellular proteins or the intracellular domains of transmembranal proteins, the proteins of the invention may be introduced into cells in a number of ways as mentioned hereinabove in (ii).

It should also be noted that the isolation, identification and characterization of the proteins of the invention may be performed using any of the well known standard screening procedures. For example, one of these screening procedures, the yeast two-hybrid procedure which was used to identify the proteins of the invention. Likewise other procedures may be employed such as affinity chromatography, DNA hybridization procedures, etc. as are well known in the art, to isolate, identify and characterize the proteins of the invention or to isolate, identify and characterize additional proteins, factors, receptors, etc. which are capable of binding to the proteins of the invention.

Moreover, the proteins found to bind to the proteins of the invention may themselves be employed, in an analogous fashion to the way in which the proteins of the invention were used as noted above and below, to isolate, identify and characterize other proteins, factors, etc. which are capable of binding to the the proteins of the invention-binding proteins and which may represent factors involved further downstream in the associated signaling process, or which may have signaling activities of their and hence would represent proteins involved in a distinct signaling process.

The DNA sequences and the encoded proteins of the invention may be produced by any standard recombinant DNA procedure (see for example, Sambrook, et al., 1989) in which suitable eukaryotic or prokaryotic host cells are transformed by appropriate eukaryotic or prokaryotic vectors containing the sequences encoding for the proteins. Accordingly, the present invention also concerns such expression vectors and transformed hosts for the production of the proteins of the invention. As mentioned above, these proteins also include their biologically active analogs, fragments and derivatives, and thus the vectors encoding them also include vectors encoding analogs and fragments of these proteins, and the transformed hosts include those producing such analogs and fragments. The derivatives of these proteins are the derivatives produced by standard modification of the proteins or their analogs or fragments, produced by the transformed hosts.

The present invention also relates to pharmaceutical compositions for modulation of the effects mediated by TRAF2. The pharmaceutical compositions comprising, as an active ingredient, any one or more of the following. (i) one or more of the DNA sequences of the

invention, or parts of them, subcloned into an appropriate expression vector; (ii) a protein according to the invention, its biologically active fragments, analogs, derivatives or a mixture thereof; (iii) a recombinant animal virus vector encoding for a protein according to the invention, its biologically active fragments, analogs or derivatives.

The invention will now be described in more detail in the following non-limiting examples and the accompanying drawings :

EXAMPLES

Materials and Methods

i) cDNA libraries

a) B-cell cDNA library

Oligo dT primed library constructed from human B cells was kindly provided by S.J. Elledge (Durfee, T. et al. (1993)). The cDNAs of the library were inserted into the XhoI site of the pACT based vector pSE1107 in fusion with GAL4 activation domain.

b) λgt10 testis cDNA library

This cDNA library from human testis was kindly provided by Dr. P. Sankhavaram. The library is a random hexanucleotide primed library with an avarage insert size of 200 to 400 bp.

ii) Yeast strains

Two yeast stains were used as hoststrains for tansformation and screening: HF7c strain that was used in the two hybrid screen and SFY526 strain that was used in the b-galactosidase assays. Both strains carry the auxotrophic markers trp1 and leu2, namely these yeast strains cannot grow in minimal synthetic medium lacking tryptophan and leucine, unless they are transformed by a plasmid carrying the wild-type versions of these genes (TRP1, LEU2). On top, the two yeast strains carry deletion mutations in their GAL4 and GAL80 genes (gal4-542 and gal80-538 mutations, respectively).

SFY526 and HF7c stains carry the lacZ reporter in their genotypes; in SFY526 strain fused to the UAS and the TATA portion of GAL1 promoter, and in HF7c three copies of the GAL4 17-mer consensus sequence and the TATA portion of the CYC1 promoter are fused to lacZ. Both GAL1 UAS and the GAL4 17-mers are responsive to the GAL4 transcriptional

activator. In addition, HF7c srain carries the HIS3 reporter fused to the UAS and the TATA portion of GAL1 promoter.

iii) Cloning of human TRAF2

The human TRAF2 was cloned by PCR from an HL60 cDNA library. The primers used were: a) 30-mer forward primer CAGGATCCTCATGGCTGCAGCTAGCGTGAC corresponding to the coding sequence of hTRAF2 starting from the codon for the first methaonine (underlined) and including a linker with BamHI site. b) 32-mer reverse primer GGTCGACTTAGAGCCCTGTCAGGTCCACAATG that includes hTRAF2 gene stop codon (underlined) and a SalI restriction site in its linker. PCR program comprised of an initial denaturation step 2 min. at 94^{0}C followed by 30 cycles of 1 min. at 94^{0}C, 1 min. at 64^{0}C, 1 min. and 40 sec. at 72^{0}C. The amplified human TRAF2 was then inserted into the BamHI - SalI sites of pGBT9 vector in conjunction with GAL 4 DNA Binding domain.

iv) Two hybrid screen of B-cell library

The two hybrid screen is a technique used in order to identify factors that are associated with a particular molecule that serves as a "bait". In the present invention TRAF2 that was cloned into the vector pGBT9, served as the bait. TRAF2 was co-expressed together with the screened B-cell cDNA library in the yeast strain HF7c. The PCR-cloned TRAF2 was a recombinant fusion with the CAL4 DNA-binding domain and the screened cDNA library was fused to the GAL4 activation domain in the pSE1107 vector. The reporter gene in HF7c was HIS3 fused to the upstream activating sequence (UAS) of the GAL1 promoter which is responsive to GAL4 transcriptional activator. Transformants that contained both pGBT9 and pSE1107 plasmids were selected for growth on plates without tryptophan and leucine. In a second step positive clones which expressed two hybrid proteins that interact with each other, and therefore activated GAL1-HIS3, were picked up from plates devoided of tryptophan, leucine and histidine and contained 50 mM 3-aminotriazol (3AT).

v) β-galactosidase assay

Positive clones picked up in the two hybride screen were subjected to lacZ color development test in SFY526 yeast cells, following Clontech Laboratories' manual. In brief, transformants were allowed to grow at 30^{0}C for 2-4 days until reaching about 2 mm in diameter, then were transferred onto Whatman filters. The filters went through a freeze/thaw treatment in order to permeabilize the cells, then soaked in a buffer (16.1 mg/ml $Na_2HPO_4 7H_2O$; 5.5 mg/ml $NaH_2PO_4 H_2O$; 0.75 mg/ml KCl; 0.75 mg/ml $MgSO_4 7H_2O$,

pH=7) containing 0.33 mg/ml X-gal and 0.35 mM β-mercaptoethanol. Colonies were monitored for development of blue color which is an indication for induction of β-galactosidase.

vi) Expression of cloned cDNAs

Two kinds of expression vectors were constructed:

a) A pUHD10-3 based vectors (constucted by M. Gossen) containing the ORF of either clone 9, 10 or 15 in fusion with the Hemeaglutinine (HA) epitope.

b) A pUHD10-3 based vector (constucted by M. Gossen) into which FLAG octapeptide sequence was introduced just in front of cloned TRAF2, hereby named FLAG/B6/TRAF2.

The constructs containing an ORF of clone 9, 10 or 15 were transfected into HeLa-Bujard cells (Gossen, M. and Bujard, M. (1992)) either alone or cotransfected with FLAG/B6/TRAF2 using standard calcium-phosphate method (Current Protocols in Molecular Biology, eds. Ausubel, F.M et al.)

vii) Luciferase assay

Typically $5x10^5$ transfected cells were harvested by washing three times with cold PBS and resuspending in 400 µl extraction buffer (0.1 M K_2HPO_4/KH_2PO_4 pH=7.8; 1 mM DTT). Lysis of the cells was achieved by three times freezing in liquid nitrogen and thawing. Cell debris was removed by centrifugation (5 min. at 10,000 x g). For the luciferase assay, 200 µl of luciferase buffer (25 mM glycylglycine, 15 mM K_2HPO_4/KH_2PO_4 pH=7.8, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, 1 mM DTT) were added to 50 µl of the lysate. Subsequently, 100 µl of 0.2 mM D-luciferine, 25 mM glycylglycine, 1 mM DTT were added to the reaction. Luciferase activity was determined by readind light emission using a Lumitron luminometer set on 10 seconds integration.

Example 1: Cloning of new clones 9, 10 and 15

cDNA library prepared from B-cells was screened for proteins that associate with TRAF2, using the two hybrid technique as described in Materials and Methods (iv). Only in transformants that expressed both TRAF2 and a protein capable of interacting with it, the GAL4 DNA-binding domain and the transcriptional activation domain were brought together. The result was the activation and expression of the reporter gene, in this case HIS3 fused to the UAS and the TATA portion of the GAL1 promoter.

The screen yield approximately 2000 clones which were able to grow on Trp-, Leu-, His- 3AT plates. DNA prepared from 165 randomly selected positive clones served for transient co-transfection of SFY526 yeast srain together with TRAF2 cloned into pGBT9 vector. Assay for b-galactosidase activity was performed on the transformed SFY526 yeast colonies as described in Materials and Methods (v). The blue color that developed was an indication for yeast colonies that contain cDNA encoding a protein or polypeptide that binds to TRAF2.

The results of the two hybride screen; the ability of the picked clones to grow on 3AT plates and to induce LacZ as measured in the color test, are summarized in Table 1. Of the positive clones checked, two were cDNAs coding for known proteins; TRAF2 itself that is capable of self-associating and forming homodimers, and the lymphotoxin beta receptor whose intacellular domains were shown to bind TRAF2. Three of the cloned cDNAs (clones 9, 10 and 15) were novel.

The positive clones were further checked in a binding specificity test, namely checked for their interaction with irrelevant baits. As shown in Table 2, clones 9 and 10 reacted only with TRAF2 and did not bind to any one of a number of irrelevant proteins checked. Clone 15, on the other hand, did not bind to MORT1, nor to the intercellular domains of the p55 and p75 TNF receptors, but did weakly bind to Lamin and to Cycline D.

In order to narrow down the region on TRAF2 molecule which interact with clones 9, 10 and 15, two additional constructs were made. One construct comprised of the N-terminal part of the TRAF2 molecule, amino-acids 1 to 221, that included the Ring finger and the zink finger motifs. The second construct included only the C-terminal part of the molecule, amino acids 222 to 501, covering the "TRAF-domain" and additional 42 amino acids. These two constructs were served as baits in two hybrid tests. The results clearly show that while clones 9, 10 and 15 did not interact with the construct comprising amino acids 1 to 221 of TRAF2 molecule, they all did bind to the C-terminal construt comprising the "TRAF domain" with the same efficiency as they bound to the full length TRAF2 molecule.

Table I: **Summary of the results of the two hybrid screen using TRAF2 as a "bait", in which clones 9, 10 and 15 were picked up.**

Growth on 50 mM 3AT	Color test (min.)	ID/name of clone, as defined by its sequencing.	Number of independent clones
+++	10 min	TRAF2	150
++	20 min	new clone number 9	6
+++	15 min	new clone number 10	2
++++	10 min	Lymphotoxin beta receptor	2
+	15 min	new clone number 15	5

Table II: **Specificity tests (interaction with irrelevant baits in the two-hybrid test)**

clone:	clone 9	clone 10	clone 15
bait			
LAMIN	-	-	+
cyclin D	-	-	+
p75-IC	-	-	-
p55-IC	-	-	-
MORT1	-	-	-
TRAF2	+++	+++	+++

Additional 500 and 700 base pair fragments corresponding to further upstream coding sequences of clone 10 were cloned by two rounds of nested PCR from λgt10 testis cDNA library. The length of the originally cloned clone 10 was about 2500 nts which was extended to yeild a clone 10 of a total length of approximately 3700 nucleotides. The extended clone 10 contained an ORF (open reading frame) of about 1250 nucleotides, starting from the most 5' end cloned nucleotide. Nested PCR of λgt10 testis cDNA library was performed using two primers corresponding to the left arm of the phage TGAGCAGCCAGTCAACACTTACGCCAAGAG and GGGTAGTCCCCACCTTTTGAGCAAGTTCAG and two antisense specific primers: for the first set of reactions AGGCCTTTGATGGGTTCTTCTCAC and GAGAGAGGTAAGGGTTCCCACATCCC; and for the second set of reactions GTCCCCATGCAGAATCCTTCGTGAGTGG and AGACAGCCCTGCTCCTTGACCAGC which are antisense specific primers based on the sequenced DNA generated by the first set of nested PCR. The two sets of nested PCR were performed under the same PCR conditions: an initial denaturation step of 2 min. at 94^{0}C followed by 30 cycles of 1 min. at 94^{0}C, 1 min. at 64^{0}C, 1 min. and 40 sec. at 72^{0}C. The first nested PCR gave a product of 800 bp that extended the length of the clone by about additional 500 nts. The second nested PCR gave a product of about 1000 bp that extended the length of the clone by additional 700 nts.

No stop codon was found also in this additional sequences, indicating that some additional 5' sequence is still missing.

Example 2: Sequencing of new clones

Three of the novel cDNA clones (clones 9, 10 and 15) were purified, amplified in E. Coli and their DNA was subject to sequence analysis. All three clones were found to be partial cDNA clones.

The total lengths of clones 9, 10 and 15 were around 2000, 3700 and 1060 base pairs, respectively.

Figs. 3, 4 and 5 show the sequenced part of the clones as follows:

Figs. 3a-b show the preliminary sequence of 521 nucleotides sequenced from the 5' end of clone 9 (a) and the deduced amino acids they encode for (b).

Figs. 4a-b show the partial and preliminary nucleotide sequence of the 5' end of clone 10 (a) and the deduced amino acids encoded thereby (b). Overall 2541 base pairs were sequenced

showing that clone 10 encodes for at least 847 amino acid long protein (the most 5' end coded part of gene was not cloned yet).

Figs. 5a-b show the entire preliminary nucleotide sequence of clone 15 sequenced from both 5' and 3' ends (a) and the deduced amino acids encoded thereby (b). Clone 15, which is a partial cDNA clone, was found to encode a 172 amino acid long protein.

Clones 9, 10 and 15, are all partial clones, which lack their most 5' end of the coding DNA sequences. The deduced amino acid sequences shown in Figs. 3b, 4b and 5b, are all started from the first nucleotide of the respective clone.

The sequence of clone 10 which was most thoroughly analyzed, encodes for a protein (NMP1) containing sequences corresponding to the I to XI conserved motifs that characterize Ser/Thr protein kinases. A computer Data base search disclosed that the protein encoded by this region show very high homology, though not identity, to the mouse MEKK protein kinase. Alignment of NMP1 with the protein kinase mMEKK's conserved motifs (marked as 1 to 11) is depicted in Fig 6. Identical amino-acid residues are denoted with vertical lines and similar amino-acids with dots.

Example 3: Expression of cloned cDNAs and their Co-immunoprecipitation with TRAF2

HeLa-Bujard cells were trasfected with TRAF2 tagged with FLAG in pUHD10-3 based expression vector and constructs containing ORF of either clone 9, 10 or 15 fused to HA epitope, as described in Materials and Methods (iv). Cells were then grown for 24 hrs. in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% calf serum with added ^{35}S-Methionine and ^{35}S-Cysteine. At the end of that incubation time cells were lysed in radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonident P-40, 1% deoxycholate, 0.1% SDS, and 1 mM EDTA; 1 ml/ $5x10^5$ cells), and the lysate was precleared by incubation with irrelevant rabbit antiserum and Protein G-Sepharose beads (Pharmacia, Sweden). Immunoprecipitation was performed by 1 hour incubation at 4°C of aliquots of the lysate with anti-FLAG (purchased from Eastman Kodak Co.) or anti-HA (clone 12CA5 kindly provided by M. Wigler (Field, J. et al. (1988)) monoclonal antibodies. The expressed proteins were analysed on SDS-PAGE gel followed by autoradiography.

The results of such experiments demonstrated that the partial cDNA clones 9, 10 and 15 encoded proteins of molecular weights around 50-65, 45 and 26 kDa respectively.

No interaction of clone 15 with TRAF2 could be detected, but the proteins encoded by clones 9 and 10 (NMP1) were co-immunoprecipitated with the TRAF2 protein. Samples of cells that were co-transfected with TRAF2 and either one of these two clones and immunoprecipitated with either anti-FLAG or anti-HA antibodies followed by analysis on SDS-PAGE as described above, displayed three bands in each lane; one band corresponding to either clone 9 or 10 encoded proteins and the other two is a doublet of 42 and 44 kDa corresponding to TRAF2 protein.

Example 4: Functional tests

Clone 10 (NMP1) was shown to block NFkB activation by two criteria: a) by gel retardation assay, and b) by reporter gene assay.

a) test by gel retardation assay: Typically 0.5-1 x 10^6 293 EBNA cells were transfected with either 10 μg of clone 10 in pcDNA3 (Fig.7 lane 1), 3 μg of pcDNA3 containing cDNA for the p75 TNF receptor (Fig. 7 lane 3), or with both clone 10 (10 μg) and p75 TNF receptor (3 μg) in Fig. 7 lane 2. In each one of the transfections the total amount of transfected DNA was brought to 15 μg with the "empty" pcDNA3 vector. As a control serve 293 EBNA cells transfected with 15 μg pcDNA3 vector alone (Fig. 7 lane 4). Cells were grown for 24 hrs in DMEM medium + 10% calf serum, then were harvested and treated according to Schreiber et al. (Schreiber, E. et al. (1989). Samples were run on 5 % polyacryleamide gel. NFkB was monitored using a set of ^{32}P-radiolabelled oligonucleotides corresponding to the NFkB binding site as probes. (The probes were GATGCCATTGGGGATTTCCTCTTT and CAGTAAAGAGGAAATCCCCAATGG).

As depicit in Fig. 7, marked inhibitory effect was observed on the induction of NF-kB by p75 TNF receptor when it was co-transfected with clone 10 cDNA. (comparing Fig.7 lanes 3 and 2).

Similar inhibitory effects were demonstrated also by another assay; a reporter gene test.

b) test by reporter gene assay:

293 EBNA cells were co-transfected with the pcDNA3 vector containing HIV LTR linked to the luciferase reporter gene, together with either pcDNA3 plasmid containing cDNA for the p75 TNF receptor alone, pcDNA3 plasmid containing clone 10 cDNA alone, or with pcDNA3 plasmid containing cDNA for the p75 TNF receptor and a pcDNA3 plasmid

containing clone 10 cDNA. Luciferase activity was monitored as described in Materials and Methods (vii).

Fig.8a shows the results of five independent transfections where cells were transfected with the reporter gene and clone 10 (black bars), or mock transfected with the "empty" pcDNA3 plasmid (grey bars). The results show that clone 10 by itself has inhibitory effects over basal NFkB activity in the transfected cells.

In another set of experiments the effect of clone 10 over activation of NFkB by p75 receptor was checked.

The results of such four independent transfections are shown in Fig. 8b. Luciferase activity in transfections including p75 receptor are marked in striated bars, and transfections including p75 receptor and clone 10 are marked in solid bars. In all cases a dramatic inhibition of NFkB activation by p75 TNF receptor was observed when the latter was co-transfected with clone 10 cDNA. The same inhibition effect by clone 10 was observed when NFkB was induced by TRAF2 instead of by p75 TNF receptor.

REFERENCES

1. Ausubel, F.M. et al. eds., Current Protocols in Molecular Biology.

2. Baeuerle, P. A., and Henkel, T. (1994) Annu Rev Immunol.

3. Bazan, J. F. (1993). Current Biology 3, 603-606.

4. Berberich, I., Shu, G. L., and Clark, E. A. (1994).. J Immunol 153, 4357-66.

5. Beutler, B., and van Huffel, C. (1994). Science 264, 667-8.

6. Blank, V., Kourilsky, P., and Israel, A. (1992). Trends Biochem. Sci 17, 135-40.

7. Boldin, M. P., Varfolomeev, E. E., Pancer, Z., Mett, I. L., Camonis, J. H., and Wallach, D. (1995). J. Biol. Chem. 270, 7795-7798.

8. Chen, C.J. et al. (1992) Ann. N.Y. Acad. Sci. 660:271-273.

9. Cheng, G., Cleary, A.M., Ye, Z-s., Hong, D.I., Lederman, S. and Baltimore, D. (1995) Science 267:1494-1498).

10.. Chinnalyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505-512.

11. Croston, G. E., Cao, Z., and Goeddel, D. V. (1995). J Biol Chem 270, 16514-7.

12. DiDonato, J. A., Mercurio, F., and Karin, M. (1995). Mol Cell Biol 15, 1302-11.

13. Durfee, T. et al. (1993) Genes Dev. 7:555-569.

14. Field, J. et al. (1988) Mol. Cell Biol. 8:2159-2165.

15. Gilmore, T. D., and Morin, P. J. (1993). Trends Genet 9, 427-33.

16. Gossen, M. and Bujard, M. (1992) PNAS 89:5547-5551.

17. Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Baxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., and Scheurich, P. (1995). Cell 83, 793-802.

18. Grilli, M., Chiu, J. J., and Lenardo, M. J. (1993). Int RevCytol.

19. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). Science 241, 42-52.

20. Hsu, H., Shu, H.-B., Pan, M.-G., and Goeddel, D. V. (1996). Cell 84, 299-308.

21. Hsu, H., Xiong, J., and Goeddel, D. V. (1995). Cell 81, 495-504.

22. Lalmanach-Girard, A. C., Chiles, T. C., Parker, D. C., and Rothstein, T. L. (1993). J Exp Med 177, 1215-1219.

23. McDonald, P. P., Cassatella, M. A., Bald, A., Maggi, E., Romagnani, S., Gruss, H. J., and Pizzolo, G. (1995). Eur J Immunol 25, 2870-6.

24. Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C., and Kieff, E. (1995). Cell 80, 389-399.

25. Rensing-Ehl, A., Hess, S., Ziegler-Heitbrock, H. W. L., Riethmüller, G., and Engelmann, H. (1995). J. Inlamm. 45, 161-174.

26. Rothe, M., Pan, M.-G., Henzel, W. J., Ayres, T. M., and Goeddel, D. V. (1995b). Cell 83, 1243-1252.

27. Rothe, M., Sarma, V., Dixit, V. M., and Goeddel, D. V. (1995a). Science 269, 1424-1427.

28. Rothe, M., Wong, S. C., Henzel, W. J., and Goeddel, D. V. (1994). Cell 78, 681-692.

29. Sambrook et al. (1989) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

30. Schreiber, E., Matthias, P., Muller, M.M. and Schaffner, W. (1989), Nuc. Acids Res. 17:6419.

31. Smith, C. A., Farrah, T., and Goodwin, R. G. (1994). Cell 76, 959-962.

32. Vandenabeele, P., Declercq, W., Beyaert, R., and Fiers, W. (1995). Trends Cell Biol. 5, 392-400.

33. Varfolomeev, E. E., Boldin, M. P., Goncharov, T. M., and Wallach, D. (1996).. J. Exp. Med. in press.

34. Wilks, A.F. et al. (1989) Proc. Natl. Acad. Sci. USA, 86:1603-1607.

35. Zhao, J.J. and Pick, L. (1993) Nature 365: 448-451.

CLAIMS:

1. A DNA sequence encoding a protein capable of binding to a tumor necrosis factor receptor-associated factor (TRAF) molecule.

2. A DNA sequence according to claim 1, wherein the TRAF molecule is TRAF2.

3. A DNA sequence according to claim 2, wherein said encoded protein binds to at least the 222-501 amino acid sequence of TRAF2.

4. A DNA sequence according to any one of claims 1 to 3, selected from the group consisting of:

(a) a cDNA sequence of the herein designated clone 9 comprising the nucleotide sequence depicted in Fig 3a.;

(b) a cDNA sequence of the herein designated clone 10 comprising the nucleotide sequence depicted in Fig 4a.;

(c) a cDNA sequence of the herein designated clone 15 comprising the nucleotide sequence depicted in Fig. 5a;

(d) a fragment of a sequence (a)-(c) which encodes a biologically active protein capable of binding to least the 222-501 amino acid sequence of TRAF2;

(e) a DNA sequence capable of hybridization to a sequence of (a)-(d) under moderately stringent conditions and which encodes a biologically active protein capable of binding to at least the 222-501 amino acid sequence of TRAF2; and

(f) a DNA sequence which is degenerate as a result of the genetic code to the DNA sequences defined in (a)-(e) and which encodes a biologically active protein capable of binding to at least the 222-501 amino acid sequence of TRAF2.

5. A DNA sequence according to any one of claims 1 to 4, selected from the sequences contained in the herein designated cDNA clones 9 and 15.

6. A DNA sequence according to any one of claims 1 to 4, which DNA encodes a protein that also modulates NF-κB activity.

7. A DNA sequence according to claim 6, selected from the sequences contained in the herein designated cDNA clone 10.

8. A DNA sequence according to claim 7, wherein said clone 10 encodes the NF-κB modulating protein 1 (herein NMP1) comprising the sequence depicted in Fig. 4b.

9. A protein encoded by a sequence according to any one of claims 1 to 8, and analogs and derivatives thereof, said protein, analogs and derivatives being capable of binding to at least the 222-501 amino acid sequence of TRAF2.

10. A protein according to claim 9, being the protein herein designated NMP1.

11. A vector comprising a DNA sequence according to any one of claims 1-10.

12. A vector according to claim 11 capable of being expressed in host cells selected from prokaryotic and eukaryotic cells.

13. Transformed prokaryotic and eukaryotic cells containing a vector according to claim 11 or 12.

14. A method for producing a protein thereof according to claim 9, which comprises growing the transformed host cells according to claim 13 under conditions suitable for the expression of said protein, effecting post-translational modification, if necessary, for extraction of said protein, and extracting said protein from the culture medium or from cell extracts of said transformed host cells.

15. Antibodies raised against a protein according to claim 9 or 10.

16. A pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a protein according to claim 9 or 10 binds, said composition comprising an effective amount of a protein according to claim 9 or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein according to claim 9 with TRAF2 or any other molecule to which a protein according to claim 9 binds.

17. A pharmaceutical composition according to claim 16, wherein said protein is NMP1.

18. A method for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a protein according to claim 9 or 10 binds, said method comprising administering to a patient in need an effective amount of a protein according to claim 9 or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein according to claim 9 with TRAF2 or any other molecule to which said protein binds.

19. A method according to claim 18, wherein said protein is NMP1.

20. A method for screening of a ligand capable of binding to a protein according to claim 9 or 10, comprising contacting an affinity chromatography matrix to which said

protein is attached with a cell extract whereby the ligand is bound to said matrix, and eluting, isolating and analyzing said ligand.

21. A method for screening of a DNA sequence coding for a ligand capable of binding to a protein according to claim 9 or 10, comprising applying the yeast two-hybrid procedure in which a sequence encoding said protein is carried by one hybrid vector and sequences from a cDNA or genomic DNA library are carried by the second hybrid vector, transforming yeast host cells with said vectors, isolating the positively transformed cells, and extracting said second hybrid vector to obtain a sequence encoding said ligand.

For the applicant

Paulina Ben-Ami

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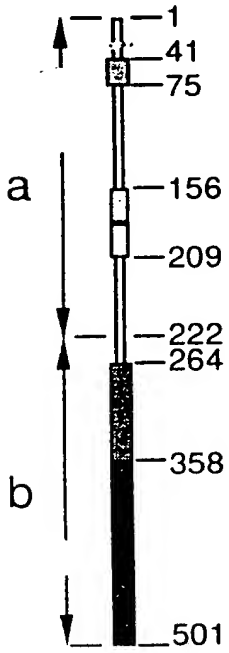
Patent Attorney

Fig 1: TRAF2 structure

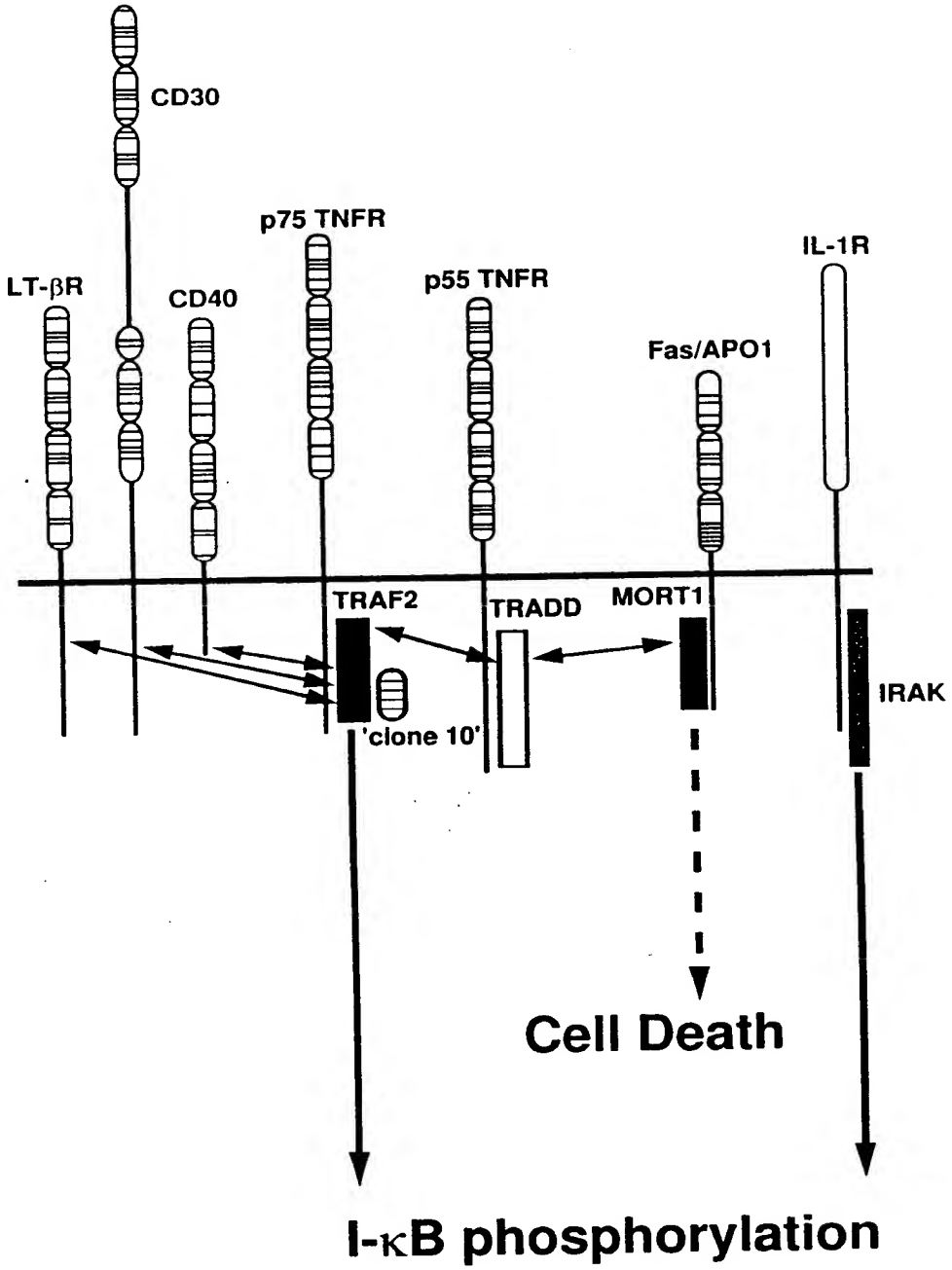


a a region that doesn't interact with clone 10 (NMP1), 9 and 15 in the 2-hybrid test

b a region that interact with these clones as strongly as the full-size TRAF2

- RING finger motif
- TFIIIA-like zinc-finger motifs
- N-TRAF
- C-TRAF

Fig 2


CD30
p75 TNFR
IL-1R
LT-βR
CD40
p55 TNFR
Fas/APO1
TRAF2
TRADD
MORT1
IRAK
'clone 10'
Cell Death
I-κB phosphorylation

```
  1  GGCGGCGCGG CGCANGCACC GGCCCGGGGA NAGGCNCCAT GAGCGGATCN
 51  CNGAACNATG ACAAAAGACA ATTTCTGCTG GAGCGACTGC TGGATGCAGT
101  GAAACAGTGC CAGATCCGCT TTNGAGGGAG AAAGGAGATT GCCTCGGATT
151  CCGACAGCAG GGTCACCTGT CTGTGTGCCC AGTTTGAAGC CGTCCTGCAG
     CATGGCTTGA AGAGGAGTCG AGGATTGGCA CTCACAGCGG CAGCGATCAA
     GCAGGCAGCG GGCTTTGcCA GCAAAACCGA AACAGAGCCC GTGTTCTgGT
301  ACTACGTGAA GGAGGTCCTC AACAAGCACG AGCTGCAGcG CTTCTACTCC
351  CTGCGCCACA TCGcCTCAGA CGTGGGCCGG GGTCGCGCCT GGCTGTGCTG
401  TGCCCTCAAC GAACACTCCC TGGAGCGCTA CCTGCACATG cTCCTGGcCG
451  ACCgCTGCAG GcTGAGCACT TTTTATGAAG ACTGGTCTTT TGTGATGGNT
501  GAAGAAAGGT CCAGTTTTgT T
```

Fig. 3a

nerated symbols 1 to: [redacted].

9.pep Length: 173 March 25, 1996 14:55 Type: P Check: 31 ..

1 RRGAXTGPGX GXMSGSXNXD KRQFLLERLL DAVKQCQIRF XGRKEIASDS

51 DSRVTCLCAQ FEAVLQHGLK RSRGLALTAA AIKQAAGFAS KTETEPVFWY

YVKEVLNKHE LQRFYSLRHI ASDVGRGRAW LCCALNEHSL ERYLHMLLAD

151 RCRLSTFYED WSFVMXEERS SFV

Fig 3b.

Fig. 4a

clone 10 DNA sequence

```
   1  AGGATCCGGG TACCATGGGG TAAGGGAAAG CGTCGCAGCA AAGCCCGGAA
  51  GAAACGGAAG AAGAAGAGCT CAAAGTCCCT GGCTCATGCA GGAGTGGCCT
 101  TGGCCAAACC CCTCCCCAGG ACCCCTGAGC AGGAGAGCTG CACCATCCCA
 151  GTGCAGGAGG ATGAGTCTCC ACTCGGCGCC CCATATGTTA GAAACACCCC
 201  GCAGTTCACC AAGCCTCTGA AGGAACCAGG CCTTGGGCAA CTCTGTTTTA
 251  AGCAGCTTGG CGAGGGCCTA CGGCCGGCTC TGCCTCGATC AGAACTCCAC
 301  AAACTGATCA GCCCCTTGCA ATGTCTGAAC CACGTGTGGA AACTGCACCA
 351  CCCCCCAGGA AGGAGGCCCC CCTGCCCCCT GCCCaCGCaC CCCTTCCCCT
 401  ATgGCAAgAC TGCCTCATCC CCTTCCCATT CCACCCTCTC CAGCCCTTGG
 451  AAACTTCACC CTCTGGAGTC CTTTCTGGGC AAAcTGGCTT GTGTAGACAG
 501  CCAGAAACCT TTGCTTGACC CACACCTGAG CAAACTGGCC TGTGTAGACA
 551  GTCCAAAGCC CCTGCCTGGC CCACATCTGG AGCCCAGCTG CCTGTCTTGT
 601  GGTGCCCATG AGAAGTTTTC CTGTGGAGGA ATCNTAGTGC ANGcTCTGCA
 651  AGGCAGCGTG AGcTCAAGCC AGGCCCACAG CatGACCAGC nTGGCCAAGA
 701  CcTGGGCAGC ACGGGGCTCC AGATCCCGGG AGCCCAGCCC CAAAACTGAG
 751  GACAACGAGG GTGTCCTGCT CACTGAGAAA CTCAAGCCAG TGGATTATGA
 801  GTACCGAGAA GAAGTCCACT GGGCCACGCA CCAGCTCCGC CTGGGCAGAG
 851  GCTCCTTCGG AGAGGTGCAC AGGATGGAGG ACAAGCAGAC TGGCTTCCAG
 901  TGCGCTGTCA AAAAGGTGCG GCTGGAAGTA TTTCGGGCAG AGGAGCTGAT
 951  GGCATGTGCA GGATTGACCT cACCCAGAAT TGTCCCTTTG TATGGAGCTG
1001  TGAGAGAAGG GCCTTgGGTC AACATCTTCA TGGAGCTGCT GGAAGGTGGC
1051  TCCCTGGGCC AGCTGGTCAA GGAGCAGGGC TGTcTCCCAG AGGACCGGGC
1101  CCTGTAcTAC CTGGGCCAGG CCcTGGAGGG TcTGGAATAC CTCCAcTCAC
1151  GAAGGATTcT GCATGGGGAC GTCaAAGCTG ACAACGTGCT CCTGTCCAGC
1201  GATGGGAGCC ACGCagCCCT CTGTGACTTT GGCCATGCTG TGTGTCTTCA
1251  ACCTGATGGC CTGGGAAAGT CCTTGCTCAC AGGGGACTAC ATCCCTGGCA
1301  CAGAGACCCA CATGGCTCCG GAGGTGGTGC TGGGCAgGAG CTGCgACgCC
1351  AAGGTGGATG TCTGGAGCag CTGCTGTATG ATGCTGCACA TGCTCAACGG
1401  CTGCCACCCC TGgACTCAGT TcTTCcGAGG GCCGcTcTgc CTCAAgATTG
1451  CCAGcGAgCC TcCgCcTGTG AgGGAgATCC CAcCCTCcTG CGCCCcTcTc
1501  ACAGCcCAGG CCATCCAAGA GGGgCTGAGG AAAGAGCCCA TCCACCGCGT
1551  GTctgCAgcG GAgtTGGGAG GGAAGGTGAA CCGGGCAcTA CAGCAAGTGG
1601  GAGGTCTGAA GAGCCCTTGG AGGGGAGAAT ATAAAGAACC AAGACATCCA
1651  CCGCCAAATC AAGCCAATTA CCACCAGACC CTCCATGCCC AGCCGAGAGA
1701  GCTTTCGCCA AGGGCCCCAG GGCCCCGGCC AGCTGAGGAG ACAACAGGCA
1751  GAGCCCCTAA GCTCCAGCCT CCTCTCCCAC CAGAGCCCCC AGAGCCAAAC
1801  AAGTCTCCTC CCTTGACTTT GAGCAAGGAG GAGTCTGGGA TGTGGGAACC
1851  CTTACCTCTG TCCTCCCTGG AGCCAGCCCT GCCAGAAACC CCAGCTCACC
1901  AGAGCGGAAA GCAACCGTCC CGGAGCAGGA ACTTGCAGCA GCTGGAAATA
1951  GAATTATTCC TCAACAGCCT GTCCCAGGCA TTTTCTCTGG AGGAGCAGGA
2001  GCAAATTCTC TCGTGCCTCA GCATCGACAG CCTCTCCCTG TCGGATGACA
2051  GTGAGAAGAA CCCATCAAAG GCCTCTCAAA GCTCGCGGGA CACCCTGAGC
2101  TCAGGCGTAC ACTCCTGGAG CAGCCAGGCC GAGGCTCGAA GCTCCAGCTG
2151  GAACATGGTG CTGGCCCGGG GGCGGCCCAC CGACACCCCA AGCTATTTCA
2201  ATGGTGTGAA AGTCCAAATA CAGTCTCTTA ATGGTGAACA CCTGCACATC
2251  CGGGAGTTCC ACCGGGTCAA AGTGGGAGAC ATCGCCACTG GCATCAGCAG
2301  CCAGATCCCA GCTGCAGCCT TCAGCTTGGT CACCCAAAGA CGGGCAGCCT
2351  GTTCCGCTAC GACATGGAGG GTGCCCAGAC TCGGGGCATC CGACCTGCAG
2401  GTGCACACTT GGCCCCCTGA TGGCAAGCTT CCGCCCTGGA GCTGGAGGGT
2451  CAAAGCCATG GCCCAGCTTG GAGGAACAAG GCCCCTAACC CTGCCCTCCA
2501  CCCGCCCGGT TCCACACTTG CCGGAAAGCA AGCCTTCCCT GCTTCGGGG
```

nerated symbols 1 to: [redacted].

x.pep Length: 849 April 2, 1996 11:48 Type: P Check: 8561 ..

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   1  RIRVPWGKGK RRSKARKKRK KKSSKSLAHA GVALAKPLPR TPEQESCTIP
  51  VQEDESPLGA PYVRNTPQFT KPLKEPGLGQ LCFKQLGEGL RPALPRSELH
      KLISPLQCLN HVWKLHHPPG RRPPCPLPTH PFPYGKTASS PSHSTLSSPW
 151  KLHPLESFLG KLACVDSQKP LLDPHLSKLA CVDSPKPLPG PHLEPSCLSC
 201  GAHEKFSCGG IXVXALQGSV SSSQAHSMTS XAKTWAARGS RSREPSPKTE
 251  DNEGVLLTEK LKPVDYEYRE EVHWATHQLR LGRGSFGEVH RMEDKQTGFQ
 301  CAVKKVRLEV FRAEELMACA GLTSPRIVPL YGAVREGPWV NIFMELLEGG
 351  SLGQLVKEQG CLPEDRALYY LGQALEGLEY LHSRRILHGD VKADNVLLSS
 401  DGSHAALCDF GHAVCLQPDG LGKSLLTGDY IPGTETHMAP EVVLGRSCDA
 451  KVDVWSSCCM MLHMLNGCHP WTQFFRGPLC LKIASEPPPV REIPPSCAPL
  01  TAQAIQEGLR KEPIHRVSAA ELGGKVNRAL QQVGGLKSPW RGEYKEPRHP
 551  PPNQANYHQT LHAQPRELSP RAPGPRPAEE TTGRAPKLQP PLPPEPPEPN
 601  KSPPLTLSKE ESGMWEPLPL SSLEPALPET PAHQSGKQPS RSRNLQQLEI
 651  ELFLNSLSQA FSLEEQEQIL SCLSIDSLSL SDDSEKNPSK ASQSSRDTLS
 701  SGVHSWSSQA EARSSSWNMV LARGRPTDTP SYFNGVKVQI QSLNGEHLHI
 751  REFHRVKVGD IATGISSQIP AAAFSLVTQR RAACSATTWR VPRLGASDLQ
 801  VHTWPPDGKL PPWSWRVKAM AQLGGTRPLT LPSTRPVPHL PESKPSLLR
```

Fig 4b.

```
   1  CTTCTTCTGT GGTCGCGGGC ACGTTTACAG CCGCAAGCAC CAGC AGC
  51  TGAAGGAGGC TTTTGANAGG CTCCTGCCCC AGGTGGAGGC GGCCCGCAAG
 101  GCCATCCGCG CCGCTCAGGT GGAGCGCTAT GTGCCCGAAC ACGAGCGATG
 151  CTGCTGGTGC CTGTGCTGCG GCTGTGAGGT GCGGGAACAC CTGAGCCATG
 201  GAAACCTGAC GGTGCTGTAC GGGGGNCTGC TGGAGCATCT GGCCAGCCCA
      GAGCACAAGA AAGCAACCAA CAAATTCTGG TGGGAGAACA AAGCTGAGGT
 301  CCAGATGAAA GAGAAGTTTC TGGTCACTCC CCAGGATTAT GCGCGATTCA
 351  AGAAATCCAT GGTGAAAGGT TTGGATTCCT ATGAAGAAAA GGAGGATAAA
 401  GTGATCAAGG AGATGGcAGC TCAGATCCGT GAGGTGGGAG CAGAGCCGAC
 451  AGGAGGTGGT TCGGTCTGTC TTAGAGcCTC AGGCAGTGCC AGACCCAGAA
 501  GAGGGGTCTT CAGCAACTAG AAAATTTGGA CCTGCCACCA GCTCCAGAGC
 551  TTGACTGGAT GGAGACAGGA CCATCTCTGA CATTCATTGG CCATCAGGAT
 601  ATACCAGGAG TTGGTAACAT CCACTCAGGT GCCACACCTC CCTGGATGAT
 651  CCAAGATGAA GAATACATTG CTGGGAACCA AGAAATAGGA CCATCCTATG
 701  AAGAATTTCT TAAAGAAAAG GAAAAACAGA AGTTGAAAAA ACTCCCCCCA
 751  GACCGAGTTG GGGCCAACTT TGATCACAGc TCCAGGACCA GTGCAGGCTG
 801  GCTGCCCTCT TTTGGGNCGC GTCTGGAATA ATGGACGCCG CTGGCAGTCC
 851  AGACATCAAC TCAAAACTGA AGCTGCAGCA ATGAAGAAGC AGTCACATAC
 901  AGAAAAAAGC TAATCATGCT CTCTACCCAA CTACCCATGA GGGTAAAAGC
 951  AAAAGTCAAC AAAACCCCTA TTATACCTTC CACCCAAATT CTTTTATCAT
1001  TGTCTTTCTT AGGAAACAGG ACATTCTCAT TCAATTGGGT TTAATAAAGT
1051  TTTATTTTTC G
```

Fig. 5a

:nerated symbols 1 to: .

r Length: 353 March 25, 1996 14:51 Type: P Check: 7923 ..

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   1  FFCGRGHVYS RKHQRQLKEA FXRLLPQVEA ARKAIRAAQV ERYVPEHERC
  51  CWCLCCGCEV REHLSHGNLT VLYGGLLEHL ASPEHKKATN KFWWENKAEV
      QMKEKFLVTP QDYARFKKSM VKGLDSYEEK EDKVIKEMAA QIREVGAEPT
 ·1   GGGSVCLRAS GSARPRRGVF SN*KIWTCHQ LQSLTGWRQD HL*HSLAIRI
 201  YQELVTSTQV PHLPG*SKMK NTLLGTKK*D HPMKNFLKKR KNRS*KNSPQ
 251  TELGPTLITA PGPVQAGCPL LGRVWNNGRR WQSRHQLKTE AAAMKKQSHT
 301  EKS*SCSLPN YP*G*KQKST KPLLYLPPKF FYHCLS*ETG HSHSIGFNKV
 351  LFF
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Fig. 5b.

Alignment of protein sequences NMP1 and mMEKK1

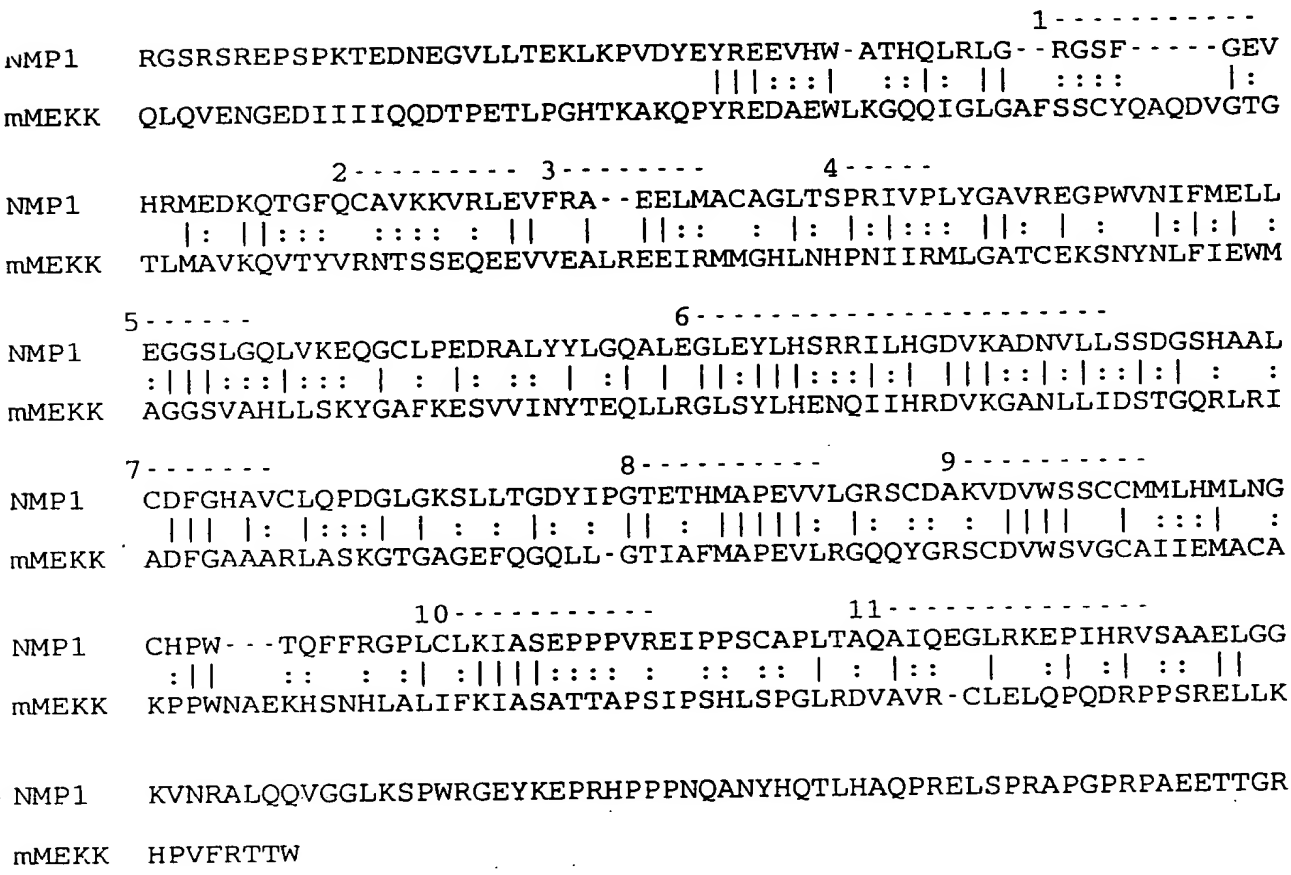


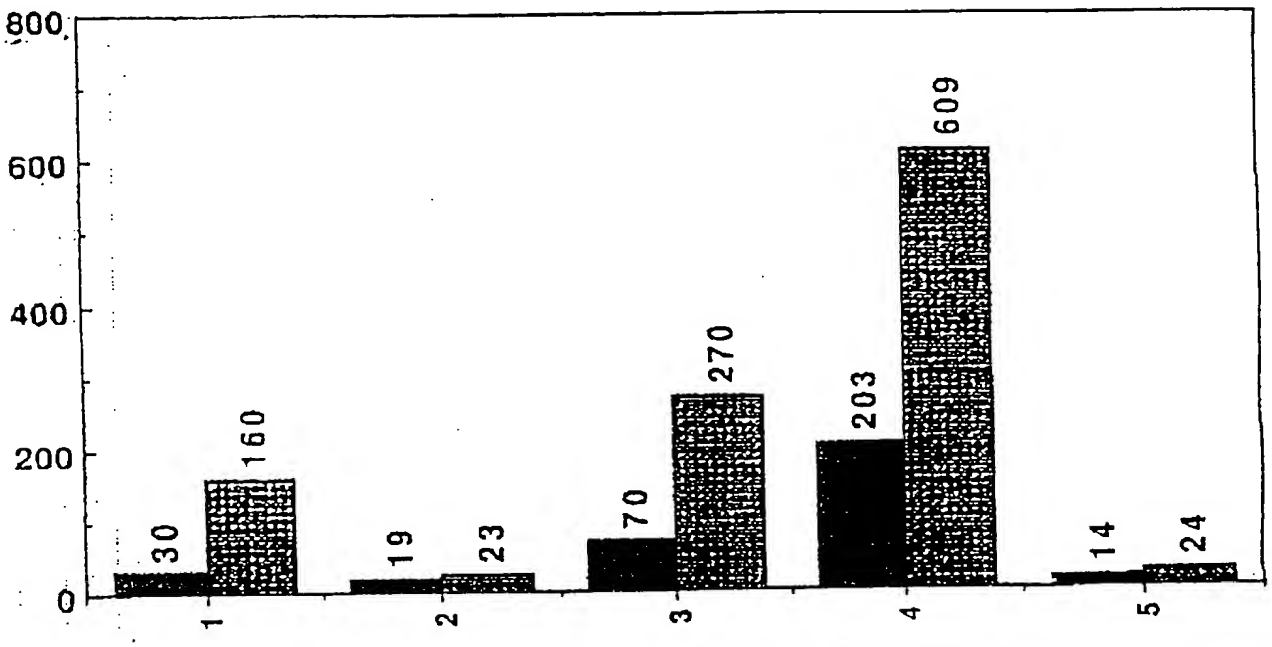
Fig. 6

1 clone 10
2 pX5 + clone 10
3 pX5
4 control

Fig. 7

Fig. 8

a)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4
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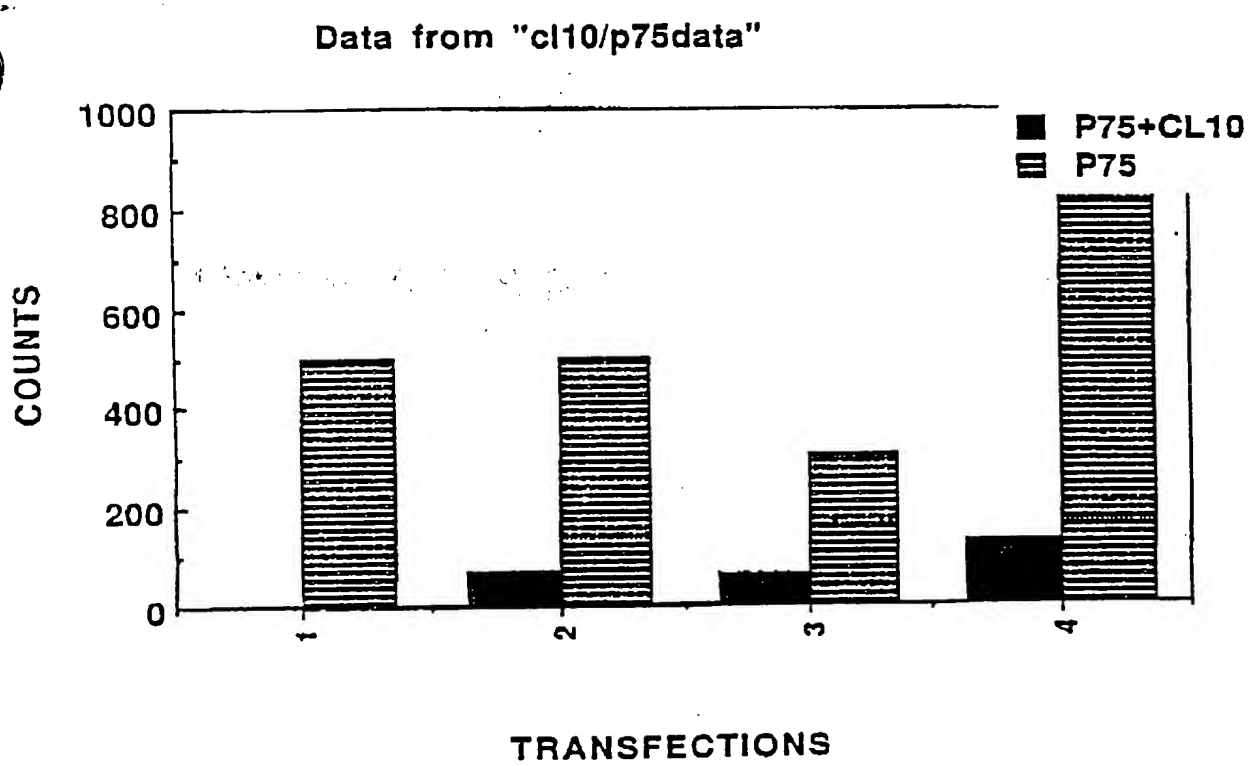
b)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4
TRANSFECTIONS